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The implications for determining whether *wnt*-5a and *wnt*-4 act as mediators of normal cell growth are relevant to the detection, diagnosis, and the treatment of breast cancer. That is, it is important ultimately to understand whether the inappropriate downregulation of certain *wnt*-genes that are spatially-temporally expressed in developing mammary glands, such as *wnt*-5a and *wnt*-4, leads to loss of growth control.

C57MG mammary epithelial cells have recently been found to endogenously express *wnt*-5a and *wnt*-4 whose RNA levels are reduced in the presence *wnt*-gene family members which transform this cell line. The intent of this proposal has been to develop new cell lines by transfecting C57MG cells with DNA *wnt*-5a and/or *wnt*-4 constructs which express antisense RNA to determine whether cell transformation will occur. In conjunction with this, new cell lines were also to be formulated by overexpressing *wnt*-5a and/or *wnt*-4 in an attempt to revert an already transformed phenotype. Preliminary results found are consistent with the importance for normal *wnt*-5a and/or RNA transcripts levels in directing C57MG mammary epithelial cell phenotype, including cell proliferation and transformation.

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Introduction

Wnt-gene family

The sixteen known members of the *wnt* family of genes express growth factor-like molecules which are thought to be implicated in mammary tumorigenesis and in early developmental events. The *wnt*-1 gene was first identified in mouse mammary tumor virus (MMTV) induced tumors occurring often in certain mouse strains (1). Analysis of these tumors revealed that MMTV proviral integration occurred in close proximity to either the 5' or 3' region of the *wnt*-1 gene locus, presumably activating *wnt*-1 through MMTV enhancer sequences (2). Since these insertions do not interrupt the protein coding sequences (3), there is expression of an intact protein. Therefore, inappropriate expression of the normal *wnt*-1 protein is implicated in cellular transformation. *Wnt*-2 and *Wnt*-3 have also been isolated from several mouse mammary tumors with activated MMTV provirus (4).

Wnt-gene mechanism of action

The mechanism of action of *wnt* genes is unknown, although several characteristics have been reported which provide insight into how their products may be important. Analysis of genomic (3) and cDNA (5) sequences has revealed that mouse *wnt*-1 encodes a cysteine rich 370 amino acid polypeptide with a leader sequence. The leader sequence, which has a potential peptidase cleavage site after amino acid 27 (6), appears to be necessary for protein function (7). Previous studies suggest that *wnt*-1 protein enters the secretory pathway, is glycosylated, and may be secreted (8), but remains strongly bound to the cell surface and/or extracellular matix (9,10), possibly to heparin (10), and is seldom found in the medium of cultured cells (9). This suggests that *wnt*-1 acts locally, possibly in an autocrine or paracrine manner. This is strengthened by the finding that mouse *wnt*-1 when transfected into 3T3 fibroblasts, which results in no obvious phenotype, can yet transform the mammary epithelial cell-line C57MG when co-cultured (11). This may be related to competence modification of cells by *wnt* signals to growth factors, including bFGF and activin (12). It has been speculated that this may occur at least in part through *wnt*-mediated gap junctional communication (13-15) as a result of influencing the expression of β-catenin to ultimately enhance $Ca2^{+}$ dependent cadherin-associated cell adhesion (16).

Wnt-genes in mammary cell differentiation and in tumorigenesis

Besides the ability of the expression of certain *wnt*s to determine cell competence during pattern formation in vertebrate development, an *in vivo* role for MMTV directed *wnt*-1 expression in mammary tumorigenesis has been directly shown in transgenic mice (17). More recently, *wnt*-3 has also been found to be transcriptionally activated by MMTV provirus in mouse mammary carcinomas (7). In addition, *wnt*-2 has been implicated in mammary tumorigenesis since it is overexpressed and amplified in transplanted virally induced tumors (4). In cell culture systems, the ectopic expression of *wnt*-1 or *wnt*-2 is able to transform certain mammary epithelial cell lines (7), including C57MGs derived from normal mammary gland of a C57 B1/6 mouse (18). However, little is known how various members of the *wnt*-gene are capable of transforming mammary epithelial cells and are involved in mammary tumorigenesis. Some investigators believe that *wnt*-1 and other transforming *wnt*-gene peptides act on endogenous *wnt*-gene signalling pathways to transform cell types.

Recent findings by Gavin and McMahon (19) have demonstrated that at least five

members of the fifteen known mouse *wnt*-genes, including *wnt*-5a and *wnt*-4, are differentially expressed during the postnatal development of the mouse mammary gland, implicating their importance in mammary development. That is, *wnt*-5a and *wnt*-4 and other *wnt*s are expressed in virgin glands and during early to mid-pregnancy. However, when pregnancy approaches term, these same genes become nonexpressive and other types of *wnt*-genes are concomittantly transcriptionally activated. That is, *wnt*-2 has recently been detected during the ductal phase of mammary gland development and reduced at the onset of pregnancy and lactation, a profile opposite to that found for *wnt*-5a and *wnt*-4 (20). **This interplay between various *wnt*-gene family members in mammary gland development may have implications for how *wnt*-1 and *wnt*-2 are able to transform cells. That is, the type of *wnt*-gene ultimately expressed may dictate the competence of the cell to respond to various growth factors and hormones and thus dictate the pattern of cell growth.**

Growth factors have been shown to cooperate with some *wnt* -peptides during early vertebrate development (12). This may also be relevant to how *wnt*-gene peptides are involved in tumorigenesis and in the *wnt*-mediated transformation of certain cell lines. In support of this, the influence of *wnt*-1, *wnt*-2, *int*-2, bFGF, EGF, and TGF-β1 on C57MG mammary epithelial cell phenotype has recently been reported (21). In addition, C57MG cells have recently been found to endogenously express *wnt*-5a and *wnt*-4 (19). Other experiments have revealed that there is a reduction of endogenous *wnt*-5a and/or *wnt*-4 RNA levels in the presence of each growth factor tested, including the *wnt*-1 and *wnt*-2 gene products (21). Furthermore, when C57MGs that were fully transformed by the ectopic expression of activated *neu* T [c-erbB2 (22)] were analyzed, it was found that transcriptional expression for each endogenous gene was virtually absent. **This suggests that endogenous *wnt*-5a and *wnt*-4 genes in C57MG mammary epithelial cells may play a functional role in determining cell growth** and that adequate RNA expression levels of both may be important in controlling cell proliferation and perhaps transformation (21).

The implications for determining whether *wnt*-5a and *wnt*-4 act as mediators of normal cell growth are relevant to the detection, diagnosis, and the treatment of breast cancer. That is, it is important ultimately to understand whether the inappropriate downregulation of certain *wnt*-genes that are spatially-temporally expressed in developing mammary glands, such as *wnt*-5a and *wnt*-4, leads to loss of growth control. This would have direct application to testing biopsied mammary tissue for loss of appropriate *wnt*-gene expression and whether the re-establishment of gene expression could result in reversal of transformation. This proposal was submitted for a **Career Development Award** to allow the opportunity to direct the laboratory toward continuing to pursue the long term goal of understanding and controlling breast cancer. The Principal Investigator is in a unique position as a member of a surgical department clinically involved in the treatment of breast cancer to examine underlying basic mechanisms.

With this in mind, a series of technical objectives was formulated, as summarized below, in order to determine the importance of *wnt*-genes in cell growth and differentiation. It was proposed that:

1. Fully transformed *neu*-T (c-erbB2) activated C57MG mammary epithelial cells would be transfected with sense *wnt*-5a and/or *wnt*-4 DNA constructs in a manner which allows for clonal selection in hygromycin B. The same was to be done for partially transformed C57MG mammary epithelial cells constitutively expressing *wnt*-1. Anchorage independent soft agar assays was to be done to determine whether the transfectants have regained cell contact growth dependence and ^{3}H-thymidine assays would be done to determine whether the transfectants have lost the ability to proliferate at confluence, which is a hallmark of transformation in this cell type.

2. To ascertain whether *wnt*-5a and *wnt*-4 genes act as repressors of cell proliferation in C57MG mammary epithelial cells, DNA constructs were to be made to express anti-sense RNA for *wnt*-5a and *wnt*-4. These constructs would be co-transfected with plasmids containing a NEO-resistant gene into the C57MG parental cell line to allow for clonal selection in G-418. Anchorage independent soft agar assays were to be done to determine whether the transfectants have gained contact growth independence. ^{3}H-thymidine assays were to be done to determine whether the transfectants have gained the ability to proliferate at confluence unlike the parental cells.

3. Since there is little information concerning the expression of *wnt*-5a and *wnt*-4 in mouse or human mammary cell lines or tumors, human and mouse cell lines as well as tumors were to be probed for the expression of *wnt*-5a and *wnt*-4 by Northern blot analysis. Assuming there is significant loss in the expression of *wnt*-5a and/or *wnt*-4 which correlates with malignant transformation, an attempt was to be made to re-express these genes to in order to re-establish growth control using nude mice.

Body

Experimental Methods

1. Transfection of transformed C57MG mammary epithelial cells not expressing *wnt*-5a or *wnt*-4 normally.

Transformed C57MG cells expressing activated V659E rat *neu* gene (provided by Oncogene Science) under the control of an MLV LTR promoter were maintained in Dulbecco's modified Eagle (DME) medium supplemented with 5% fetal calf serum and 5% bovine calf serum (HyClone), and 250 ug/ml G-418 (Gibco). The cells were transfected with the mammalian expression vector pRSV ligated to*wnt*-5a or *wnt*-4 (provided by Andrew McMahon, Harvard) in the sense orientation using lipofection as described (23). The cells were co-transfected with the SV2HYG mammalian expression vector carrying the gene for hygromycin B resistance, and resistant colonies selected and expanded into cell lines. RNA was extracted (24) and cells expressing *wnt*-5a and *wnt*-4 verified by Northern blot analysis using specific hybridization probes for *wnt*-5a and *wnt*-4 and compared to those cells only transfected with the SV2HYG vector. The same technique was used to transfect *wnt*-1 expressing C57MG cells (provided by Jackie Papkoff, Sugen, California) which are maintained normally in Dulbecco's modified Eagle medium as above also supplemented with 250 ug/ml G-418 (Gibco) with pRSV*wnt*-5a and/or pRSV*wnt*-4.

RNA isolation and Northern Blot Analysis

Total cellular RNA was isolated from multiple dishes of confluent cells (24). Twenty ug of total RNA was analyzed when probing for transfected gene expression. To ascertain effects on endogenous levels of *wnt* RNA tarnscripts, it was necessary to select for poly (A+) RNA using an oligo d(T) cellulose column. Two ug of each poly (A+) RNA sample were separated on a 1.2% agarose formaldehyde gel followed by transfer to a Hybond-N (Amersham) membrane. The membranes were cross-linked (Stratagene) and prehybridized for 3-6 hours at 61^{o}C, and then hybridized at 61^{o}C overnight with labeled riboprobes made with a Ribosystem II kit (Promega). The riboprobe vectors containing sequences for *wnt*-4 or *wnt*-5a (McMahon, Harvard) have been described (19). The hybridization/prehybridization solution consisted of 50% formamide, 4X SSPE, 0.2 mg/ml sheared salmon sperm DNA, 2.5X Denhardt's, and 1% sodium dodecyl sulfate (SDS). Membranes were washed at room temperature twice in 2X SSC, 1% SDS, followed by several washes in 0.1X SSC, 0.1% SDS at 65^{o}C.

Cell Morphology Assays

Cells lines were maintained in G-418 and hygromycin B and grown to confluence. C57MG/*wnt*-1, C57MG/neu T, C57MG, C57MG/*wnt*-1/ *wnt*-5a and/or *wnt*-4, and C57MG/neu T/*wnt*-5a and/or *wnt*-4 cells were plated at a density of $5x10^5$ cells per 10 cm tissue culture dish and photographed for phenotype comparison.

2. **Transfection of C57MG mammary epithelial cells with antisense constructs for *wnt*-5a or *wnt*-4.**

The cells were initially maintained in Dulbecco's modified Eagle's medium until mid-log phase and transfected by lipofection (23) with either pRSV*wnt*-5a and/or *wnt*-4 DNA mammalian expression vectors ligated in the antisense orientation. The cells were co-transfected with the mammalian expression vector SV2NEO to allow for clonal selection in G-418. Individual resistant colonies were selected and expanded into cell lines. To determine whether the antisense RNA leads to specific loss of *wnt*-5a or *wnt*-4 transcripts, Northern blots were done.

RNA isolation and Northern Blot Analysis

Total cellular RNA was isolated from multiple dishes of confluent cells (24). Twenty ug of total RNA was analyzed when probing for transfected gene expression. To ascertain effects on endogenous levels of *wnt* RNA tarnscripts, it was necessary to select for poly (A+) RNA using an oligo d(T) cellulose column (62). Two ug of each poly (A+) RNA sample were separated on a 1.2% agarose formaldehyde gel followed by transfer to a Hybond-N (Amersham) membrane. The membranes were cross-linked (Stratagene) and prehybridized for 3-6 hours at 61°C, and then hybridized at 61°C overnight with labeled riboprobes made with a Ribosystem II kit (Promega). The riboprobe vectors containing sequences for *wnt*-4 or *wnt*-5a (McMahon, Harvard) have been described (19). The hybridization/prehybridization solution consisted of 50% formamide, 4X SSPE, 0.2 mg/ml sheared salmon sperm DNA, 2.5X Denhardt's, and 1% sodium dodecyl sulfate (SDS). Membranes were washed at room temperature twice in 2X SSC, 1% SDS, followed by several washes in 0.1X SSC, 0.1% SDS at 65°C.

Cell Morphology Assays

Cells lines were maintained in G-418 and hygromycin B and grown to confluence. C57MG/*wnt*-1, C57MG/neu T, C57MG, C57MG/*wnt*-1/ *wnt*-5a and/or *wnt*-4, and C57MG/neu T/*wnt*-5a and/or *wnt*-4 cells were plated at a density of $5x10^5$ cells per 10 cm tissue culture dish and photographed for phenotype comparison.

Preliminary Results

Preliminary studies indicate that transfecting *wnt*-1 expressing C57MG cells with a construct which constitutively expresses *wnt*-4 partially rescues the *wnt*-1 mediated transformed phenotype based on morphologic assays as indicated in the microphotographs in Fig 1. It was also found that C57MG cells fully transformed with *neu* T also revert to a more normal appearing phenotype by morphologic criteria particularly when the cells are transfected simultaneously with both *wnt*-5a and *wnt*-4 as shown in Fig 2.

To determine whether the endogenous expression of *wnt*-4 and/or *wnt*-5a are important for normal cell growth, DNA constructs were made which allow for the expression of *wnt*-4 or *wnt*-5a antisense RNA. By morphologic criteria, antisense *wnt*-5a dramatically alters cell phenotype, as noted in Fig 3 (b) and (c) when compared to the normal parental line as shown in Fig 3 (a) and Fig 1 (a). This change in phenotype mimics the transformed phenotype observed in the presence of the overexpression of *wnt*-1 in this cell line, as shown in Fig 1 (b). Furthermore, cells which are expressing antisense *wnt*-5a have the ability to continue to grow after confluence unlike the parental

cell line. This change in phenotype becomes even more exaggerated in those cell lines which are constitutively expressing antisense *wnt*-5a and *wnt*-4 simultaneously.

These preliminary findings suggest the importance of the presence of *wnt*-5a and/or RNA transcripts in directing C57MG mammary epithelial cell phenotype and cell proliferation, and may be the underlying mechanism for how *wnt*-1 is able to transform the cell phenotype. These results are consistent with the proposed hypothesis that *wnt*-5a and/or *wnt*-4 control normal cell growth and provide a basis for continuing this line of research for understanding mammary cancer etiology. We have fulfilled the goals proposed for the first 12 months of the grant proposal by establishing all the stably transfected cell lines outlined in the proposal and now are characterizing these cell lines by various assays. We are currently or will be soon studying these cell lines with anchorage independent assays, 3H-thymidine incorporation, flow cytometry, and the use of nude mice, as described in the proposal.

Conclusions

The results indicate to date that *wnt*-4 and *wnt*-5a gene expression is necessary for normal cell growth of C57MG mammary epithelial cells. This is an important finding since *wnt*-4 and *wnt*-5a are differentially expressed in normal developing mouse mammary gland. Considering that *wnt*-1 transgenic mice develop mammary gland hyperplasias and adenocarcinomas and *wnt*-1 expressing C57MG cells transform, it becomes critical to understand the *wnt*-1 mechanism of action. **One conclusion to draw from the results already published by this author and from the preliminary results derived from this proposal, is that it appears that *wnt*-1 transforms cells by altering the normal expression of other *wnt*-gene family members, including *wnt*-5a and *wnt*-4.** It may be that whatever, *wnt*-gene family member predominates in a cell or collection of cells dictates the cell phenotype. This has important implications to determine *wnt*-gene expression in breast cancer. Interestingly, this proposal included ascertaining *wnt*-5a and *wnt*-4 RNA levels in human breast cancer tissue and cell lines. Published studies have now shown that *wnt*-5a and *wnt*-4 is minimally expressed, if at all, in human breast cancer cell lines (25, 26). Tissue from normal human breast when compared to benign breast tumors and malignant breast tumors for *wnt*-5a and *wnt*-4 gene expression gives conflicting results, but generally follows the pattern that benign tissue *wnt*-5a and *wnt*-4 expression is significantly increased while that for malignant tumors is decreased. The important point is that *wnt*-5a and *wnt*-4 gene expression is altered depending on the state of cell growth.

We are planning to extend these results by searching for genes that may be transcriptionally regulated in the presence or absence of *wnt*-1, and *wnt*-4 or *wnt*-5a using PCR-based RNA fingerprinting by differential display (27). This technique should provide for a systematic approach for uncovering *wnt*-mediated gene transcription. The cell lines already established in this proposal will be of particular use for minimizing false positives in searching for specific genes controlled by *wnt*-transcription.

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Appendices

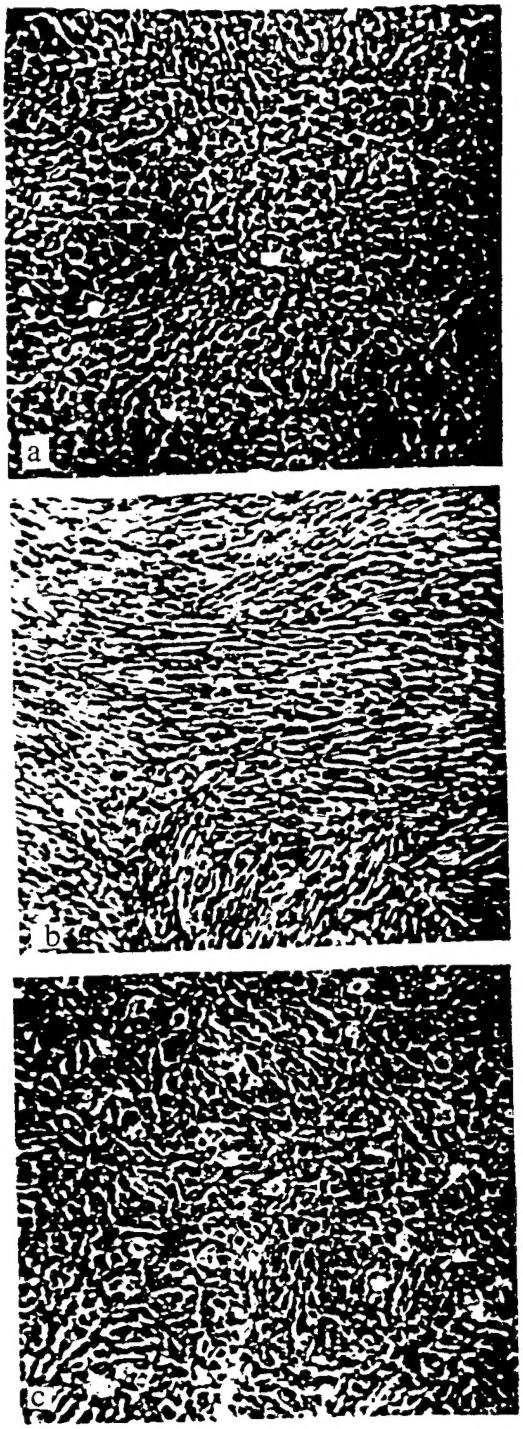

Fig 1. Photomicrographs of a morphology assay showing C57MG mammary epithelial cells at confluence which compare (a) normal parental cells with (b) *wnt*-1 expressing cells and with (c) *wnt*-1 expressing that have been co-transfected with *wnt*-4 in the sense orientation.

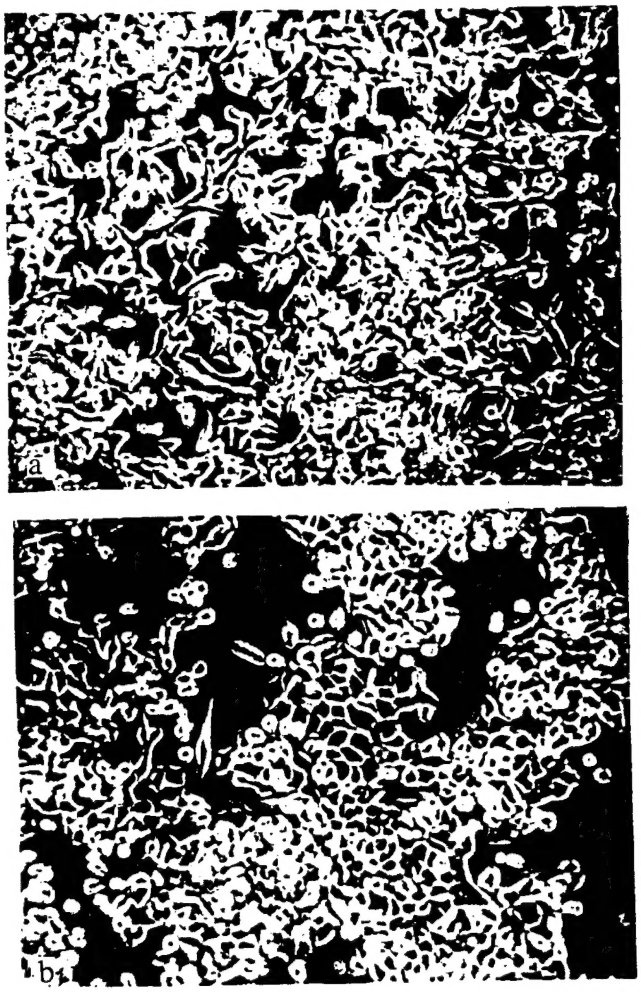

Fig. 2 Photomicrographs of C57MG mammary epithelial cells which compare (a) cells which are expressing rat *neu* T and are fully transformed to (b) cells which are expressing rat *neu*T, and are co-transfected with *wnt*-5a and *wnt*-4 in the sense orientation.

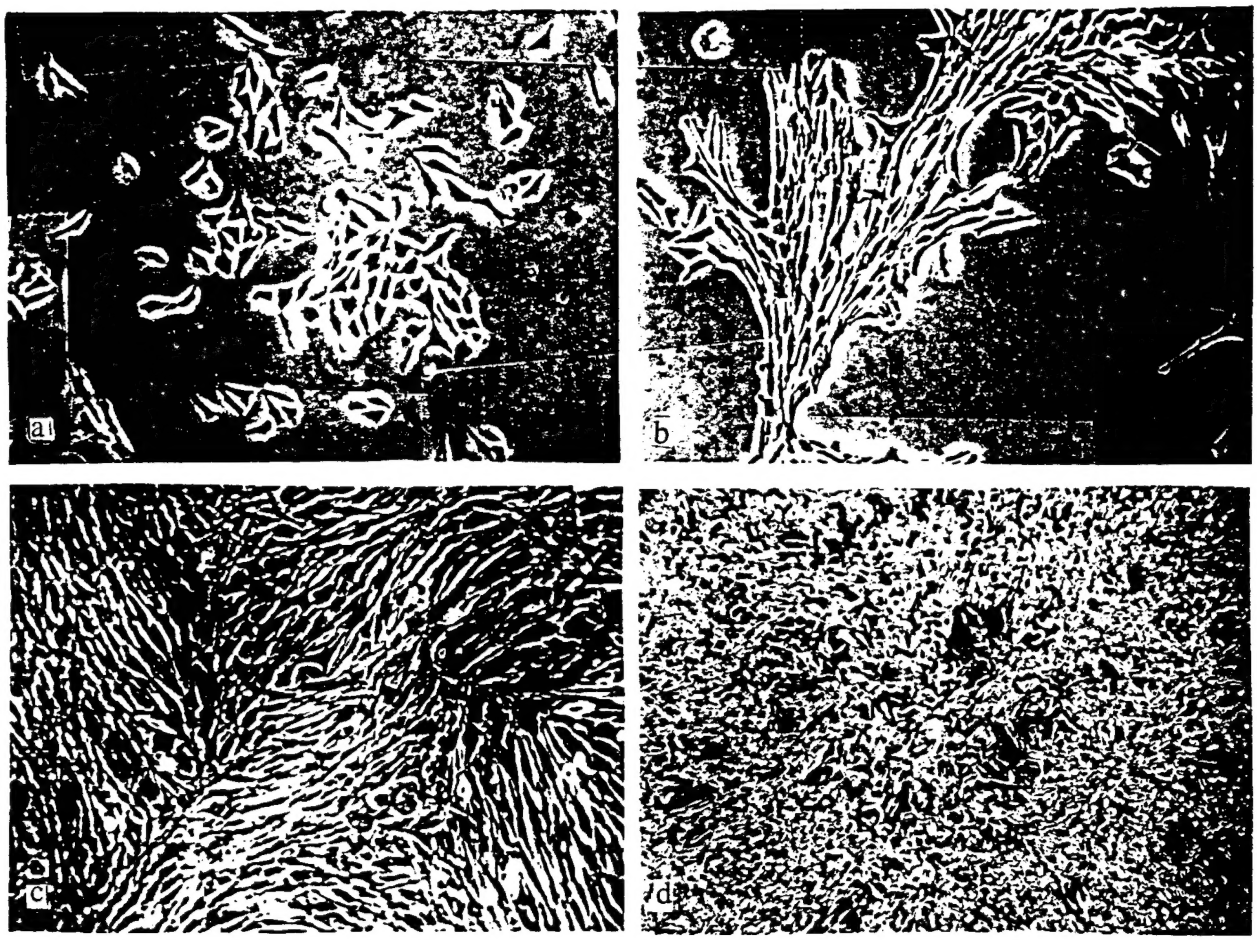

Fig 3. Photomicrographs of C57MG mammary epithelial cells which compare (a) normal parental cells at subconfluence to (b) cells which have been transfected with *wnt*-5a in the antisense orientation and to (c) cells transfected with antisense *wnt*-5a at confluence. Compare (c) with *Fig 1 (a)*. The photomicrograph represented in (d) are cells at confluence which have been transfected with both *wnt*-5a and *wnt*-4 in the antisense orientation, which can be compared to *Fig 2 (a)*.